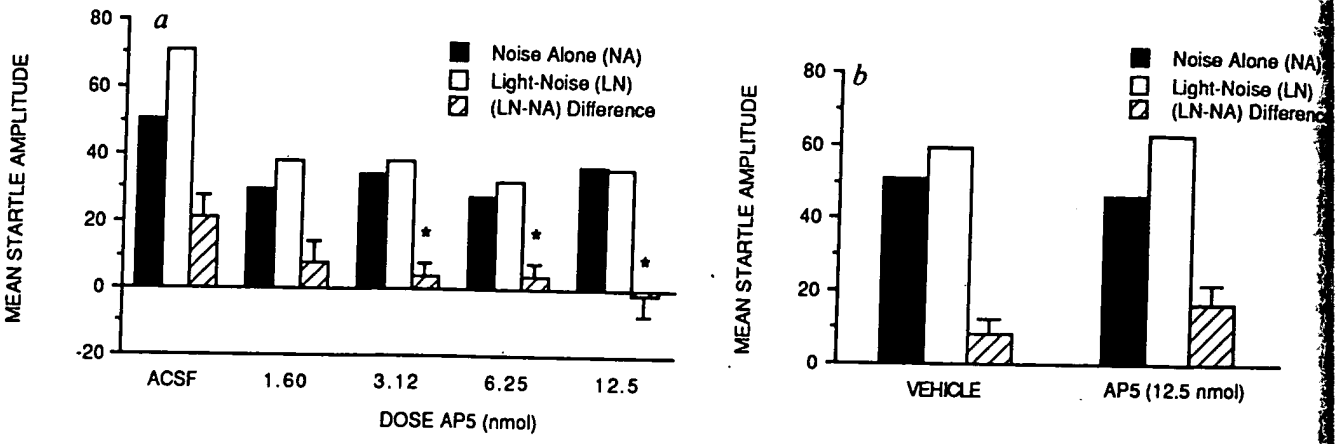

FIG. 3 *a*, Infusion of AP5 into the amygdala immediately before a shorter training procedure (a 15-min session consisting of five light (conditioned stimulus)–footshock (unconditioned stimulus) pairings, with a 2-min interstimulus interval) dose-dependently blocked acquisition of conditioned fear (F_{linear} (1, 23) = 7.43, $P < 0.01$), and significantly reduced potentiated startle at doses of 3.12 nmol and greater. *b*, The highest effective dose of AP5 (12.5 nmol) used to block acquisition of conditioned fear in the short training procedure did not attenuate the expression of fear-potentiated startle compared with vehicle controls, when infused into the amygdala immediately before testing. Hence the overall difference between the light-noise and noise-alone trials was statistically significant ($F(1, 9) = 11.97$, $P < 0.01$) but there was no group by trial-type interaction ($F(1, 9) = 1.18$, not significant) and significant potentiated startle occurred in the AP5 group ($t(5) = 2.91$, $P < 0.03$).

study attenuated potentiated startle through actions on a substrate ≤1.0 mm from the basolateral nucleus. Therefore, two rats with one or both cannula tips located beyond that distance did not show a decrease in fear conditioning, even when normally effective doses were infused.

The finding that NMDA receptor antagonists infused into the amygdala blocked fear-potentiated startle indicates that an NMDA-mediated process at that level is critical for the acquisition of conditioned fear. The many demonstrations that NMDA antagonists also block induction of long-term potentiation (LTP), and the finding in intracellular studies that LTP occurs in the amygdala[27], suggest that an NMDA-dependent form of LTP in the amygdala might underlie fear conditioning.

Investigations of the physiological and biochemical mechanisms of learning and memory have mainly made use of *in vitro* preparations. The results of the present study, suggesting the existence of an NMDA-dependent process in the amygdala subserving fear conditioning, show that it may now be possible to use a behavioural measure, with a defined neural circuit tightly coupled to the possible site of plasticity, to evaluate the role of these mechanisms *in vivo*. □

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Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation

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IN both vertebrates and invertebrates, long-term memory differs from short-term in requiring protein synthesis during training[1,2]. Studies of the gill and siphon withdrawal reflex in *Aplysia* indicate that similar requirements can be demonstrated at the level of sensory and motor neurons which may participate in memory storage. A single application of serotonin[3], a transmitter that mediates sensitization, to individual sensory and motor cells in dissociated cell cultures leads to enhanced transmitter release from the sensory neurons that is independent of new macromolecular synthesis. Five applications of serotonin cause a long-term enhancement, lasting one or more days, which requires translation and transcription[2,3]. Prolonged application or intracellular injection into the sensory neuron of cyclic AMP, a second messenger for the action of serotonin, also produce long-term increases in synaptic strength[4,5], suggesting that some of the gene products important for long-term facilitation are cAMP-inducible. In eukaryotic cells, most cAMP-inducible genes so far studied are activated by the cAMP-dependent protein kinase (A kinase), which

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phosphorylates transcription factors that bind the cAMP-responsive element TGACGTCA. The cAMP-responsive element (CRE) binds a protein dimer of relative molecular mass 43,000, the CRE-binding protein (CREBP), which has been purified and shown to increase transcription when phosphorylated by the A kinase[6–11]. Here we show that extracts of the *Aplysia* central nervous system and extracts of sensory neurons contain a set of proteins, including one with properties similar to mammalian CREBPs, that specifically bind the mammalian CRE sequence. Microinjection of the CRE sequence into the nucleus of a sensory neuron selectively blocks the serotonin-induced long-term increase in synaptic strength, without affecting short-term facilitation. Taken together, these observations suggest that one or more CREB-like transcriptional activators are required for long-term facilitation.

We first determined whether the *Aplysia* central nervous system (CNS) contains a CREBP-like protein. Gel mobility shift assays on extracts of *Aplysia* CNS using a rat somatostatin CRE probe, gave three specifically retarded bands (Fig. 1*a*). The same number of retarded bands was seen when the CRE sequence from the vasoactive intestinal protein (VIP) gene was used as the probe (data not shown). A similar mobility shift pattern was also observed with extracts from *Aplysia* sensory neurons. Moreover, using antibody to the mouse CREBP[12], we have found cross-reactivity with a protein of relative molecular mass 43,000 (43K) from *Aplysia* CNS extract which is highly enriched after partial purification on a CRE affinity column (data, including a western blot, not shown).

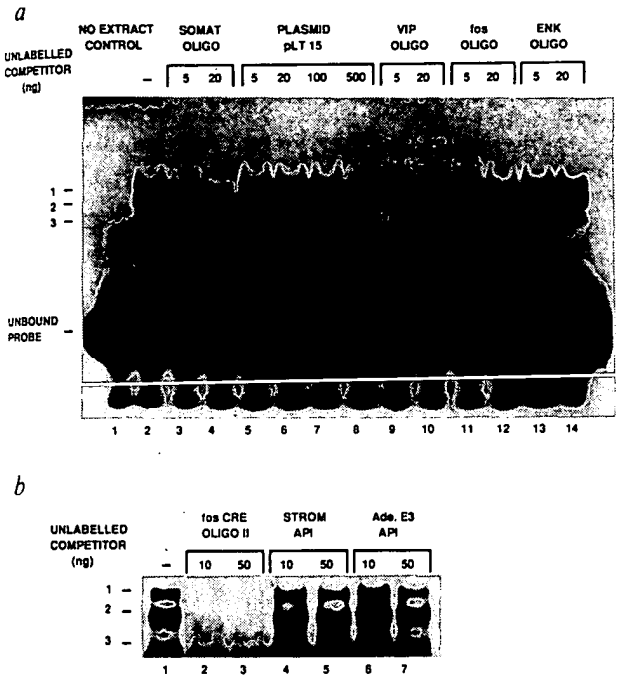

FIG. 1 Specific binding of *Aplysia* CNS extract to CRE of rat somatostatin gene. *a*, Protein binding to CRE was performed by gel-retardation assay. The positions of the free and of the three retarded bands are shown. The probe, and the probe plus extract in the absence of competitor are in lanes 1, 2. The three retarded bands can be competed out specifically by non-radioactive somatostatin CRE (lanes 3, 4) but not by linear nonspecific plasmid DNA (lanes 5–8). The competition by CRE sequence from VIP (−99 to −58), *fos* (−330 to −276), and enkephalin (−114 to −70) is shown in lanes 9–14 (ref. 9). *b*, AP-1 sequence does not compete with the binding of the protein to CRE. Lane 1 shows the binding of the *Aplysia* CNS extract to somatostatin CRE in the absence of competitor. The second CRE sequence from *fos* gene (−78 to −48) (ref. 23) competes well with binding (lanes 2, 3). But the AP1 sequence from rat stromolysin (strom) (−84 to −57) (ref. 24) or the adenovirus E3 gene (Ade. E3) (−109 to −79) (ref. 17) does not compete to the same extent as the CRE sequences at the concentrations tested (lanes 4–7).

METHODS. *Aplysia* CNS (abdominal, pleural and pedal ganglia) from 100–120 g animals were dissected from isotonic $MgCl_2$-anaesthetized animals. The total-protein extract from the desheathed ganglia was prepared as described previously[25]. Two complementary oligonucleotides corresponding to the rat somatostatin (CRE (−75 to −26) containing *Bam*H1 and *Pst*I sites were synthesized, hybridized, and cloned into vector PKSM13$^+$. The assay probe was prepared by digesting the plasmid with *Eco*RI, filling the ends with radioactive dATP and dTTP, followed by *Bam*H1 digestion and purification on an acrylamide gel. Synthetic oligonucleotides were used for competition assay. The assay was carried out as described previously[26] using ~5 μg protein, ~0.2–0.5 ng of probe, and 1 μg each of salmon sperm DNA and poly(dI-dC)·poly(dI-dC) as non-specific competitor in a 20 μl reaction volume.

Binding of *Aplysia* proteins to somatostatin CRE is sequence-specific. It is effectively inhibited to varying degrees by non-radioactive oligonucleotides containing the CRE sequences from somatostatin, VIP, *fos* or enkephalin (Fig. 1*a*). The differential inhibition seen may reflect either differences in the affinity of the protein for slightly different CRE sequences, or the contribution to binding affinity of the distinctive CRE flanking sequences[13,14]. By contrast, nonspecific linear plasmid DNA (PKSM13$^+$) did not compete with binding, and only at a very high concentration (Fig. 1*a*) was there some nonspecific decrease in binding. Two mutant oligonucleotides (TGAAGCCA and CTTAAGTG) with the same flanking sequences as the somatostatin probe also failed to compete with the binding. Moreover, the two AP-1 sequences (TGAGTCA) differing from the CRE sequence in only one nucleotide compete weakly at the concentrations we used (Fig. 1*b*). Similar weak competition has been previously reported[8,14–16].

To characterize the DNA-protein interaction at the CRE site, we carried out DNase I footprinting in parallel nuclear extracts derived from *Aplysia* CNS and HeLa cells. Addition of HeLa nuclear extract gave two specific retarded bands and one non-specific band in the gel shift assay (data not shown). Both the specific retarded bands are protected from DNase I digestion at and around the CRE sequence as are the three retarded bands of *Aplysia* CNS extract (Fig. 2). Comparison of the two hypersensitive sites shows that the DNase protection by HeLa extract covers more nucleotides than the *Aplysia* extract.

Next we determined whether a protein like the CREBP is involved in long-term facilitation, which is transcription dependent and initiated by cAMP. As the site of plasticity for this long-term increase in transmitter release resides within the sensory neuron[17], we isolated sensory and motor neuron pairs in culture[18], and injected double-stranded somatostatin CRE sequence into the nucleus of the sensory neuron (Fig. 3). After injection, we treated the culture dish with five exposures of serotonin and monitored long-term facilitation by examining the connection 24 h later. If the injected CRE sequence competed for binding of a CREBP-like protein, this protein might be prevented from activating the cAMP-inducible genes, despite the increase in cAMP produced by serotonin. A blockade of long-term facilitation might result (Fig. 3*b*).

We found that cells injected with control (mutant) oligonucleotides showed an increase in synaptic strength one day after serotonin treatment (31% ± 14% s.e.m., $n = 7$), which was comparable to that occurring in uninjected cells (38% ± 13% s.e.m., $n = 43$) (Fig. 4*a*, *b*). By contrast, cells injected with somatostatin CRE did not show an increase in the excitatory postsynaptic potential (e.p.s.p.) 24 h after serotonin treatment and were comparable to cells not exposed to serotonin and significantly different from control cells exposed to serotonin (7% ± 8.5% s.e.m., $n = 19$, $P < 0.05$). Whereas the CRE oligonucleotides blocked long-term facilitation, these oligonucleotides had no effect on short-term facilitation, as tested either immediately after injection (data not shown) or 24 h later (Fig. 4*c*). Moreover, the effect on long-term facilitation was titratable. Injection of 20-fold less CRE blocked long-term facilitation only partially ($n = 4$).

To rule out a nonspecific reduction in the long-term facilitation attributable to a general effect on transcription, due to either

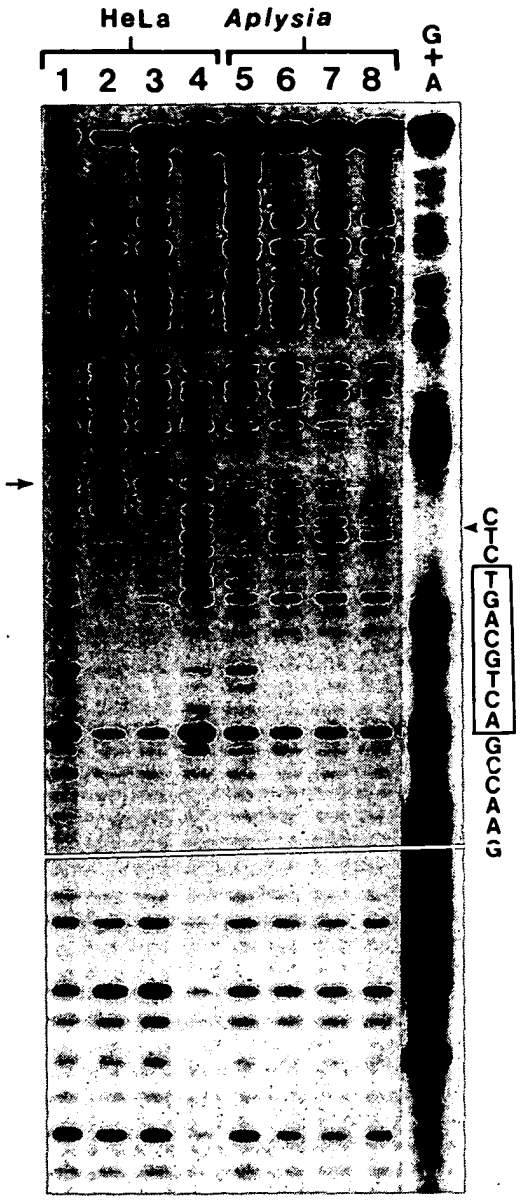

FIG. 2 Analysis of the protein–DNA interaction at the CRE site. DNase I footprinting of the coding strand of the rat somatostatin CRE with HeLa nuclear and *Aplysia* CNS extract. An autoradiograph of the sequencing gel is shown. Two retarded bands were seen using HeLa nuclear extract. The footprint of the unbound (lanes 1), nonspecifically bound (lane 4), and the two retarded bands (lanes 2, 3) are shown. The footprint of the unbound band (lane 5) and three retarded bands (lanes 6–8) with the partially purified heparin-agarose fraction of the *Aplysia* CNS extract is shown. The DNase I hypersensitive sites are shown by arrows. The same probe was independently cleaved with piperidin (A + G ladder) for sequence alignment of the CRE motif and is shown at the right.

METHODS. The footprinting reactions were prepared in a total volume of 50 μl containing 1–2 ng of end-labelled DNA probe as described previously[25]. The probe was obtained from PKSM13$^+$ plasmid containing the CRE sequence, by digesting with *Xhol*, filling the end with ^{32}P nucleotides and Klenow polymerase followed by a second digestion at the *Bam*H1 site. The fragment containing the CRE sequence was purified in an acrylamide gel. About 20 μl protein was incubated with 1–2 ng of the probe in the presence of nonspecific competitor for 30 min at room temperature. After DNase I digestion (Pharmacia; 10 units for 30 s), the free and retarded bands were separated on a low-ionic-strength acrylamide gel. The bands were detected by autoradiography, excised and eluted in 0.5 M ammonium acetate, 1 mM EDTA and 0.1% SDS overnight at 37 °C. The eluted DNA was run on a 6% sequencing gel.

titration of a general transcription factor(s) or some structural features specific to enhancer sequences, we also injected two other known enhancer sequences: (1) the consensus heat-shock element (HSE); and (2) the NF-$_\kappa$B enhancers[19,20]. Neither injection of HSE, whose sequence is conserved from human to yeast, nor injection of NF-$_\kappa$B enhancer, affected the increase in synaptic strength seen 24 h after serotonin treatment (Fig. 4*b*). Gel shift assay shows that a heat-shock transcription factor is present in sensory neuron extracts and its binding can be induced by heat treatment.

To ensure that the CRE injection did not simply produce its blockade by a general inhibition of transcription, we injected

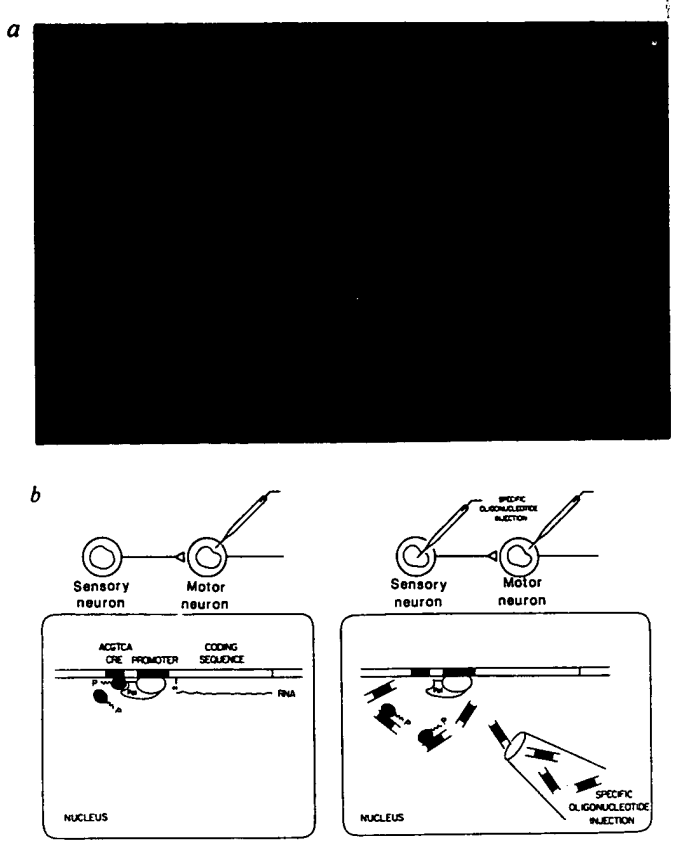

FIG. 3 *Aplysia* sensory-motor neuron culture system used to study the inhibition of long-term facilitation by CRE oligonucleotide injection. *a*, Phase contrast photomicrograph of a culture of motor neuron L7 and two sensory neurons. One of the sensory neurons was injected (SN2) with the specific oligonucleotide, whereas the other one was uninjected or injected with a control oligonucleotide. *b*, A schematic representation of the experimental design to study the effect of CRE injection.

METHODS. One to three pleural sensory neurons from a 100 g animal and an L7 motor neuron from a juvenile animal were co-cultured as described previously[18,27]. After 4–5 days in culture, the amplitude of the e.p.s.p. evoked in L7 by each sensory neuron was measured using extracellular electrodes to stimulate the sensory cell. One of the sensory cells was pressure-injected with 10–30 pl of 5 μg ml^{-1} double-stranded somatostatin CRE oligonucleotides (about 10^6 copies) in diethylpyrocarbonate-treated 0.5 M potassium acetate, 10 mM Tris HCl (pH 7.6) and 0.2% Fast Green. The other cell was either injected with control oligonucleotide or was left uninjected. After recording, the cultures were treated with 5 μM serotonin to induce long-term facilitation[2]. After 24 h the e.p.s.p. was measured. The nucleotide sequences of oligonucleotides used in these studies were: CRE, 5′-GGCCTCCTTGGCTGACGTCAGAGAGAGAGTTCTGCA-3′; Mutant, 5′-GGCCTCCTTGGCCTTAAGTGGAGAGAGAGTTCTGCA-3′; NF-$_\kappa$B, 5′-GATCTCAACAGAGGGGACTTTCCGAGGCCA-3′; HSE, 5′-GGAGCGCGCCTCGAATGTTCTAGAAAAGGCTGCA-3′. Oligonucleotides were synthesized with a *Pst*I overhang, except NF-$_\kappa$B which has a GATC overhang. Both the complementary strands were purified and hybridized to each other. After hybridization, the double-stranded oligonucleotides were phenol-extracted and then precipitated with ethanol before use.

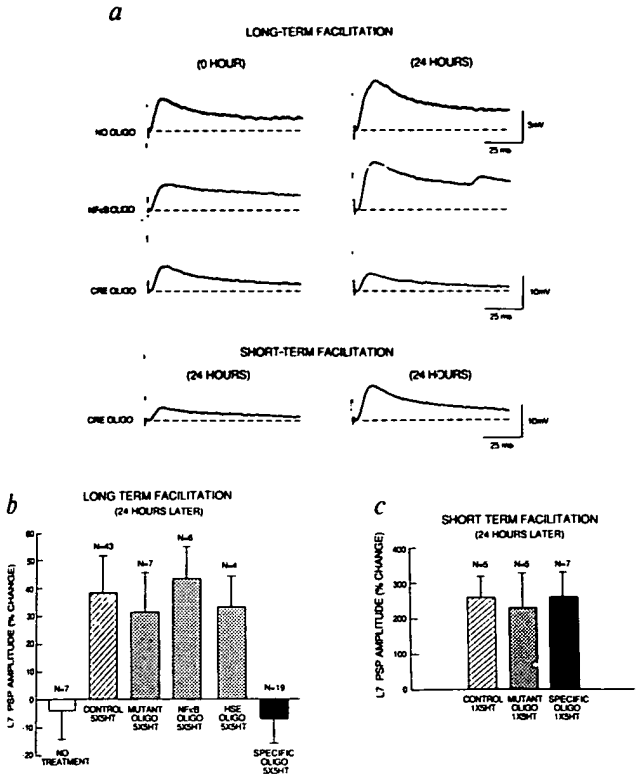

FIG. 4 Injection of CRE oligonucleotides blocks serotonin-induced long-term facilitation. *a*, The e.p.s.p. on to the L7 motor neuron at 0 h (before serotonin treatment) is compared with the first e.p.s.p. 24 h after the treatment. Injection of the control oligonucleotide does not affect the increase in the e.p.s.p. whereas CRE injection blocks the increase 24 h after treatment. Short-term facilitation in the presence of serotonin is not affected by CRE oligonucleotide injection 24 h after the long-term training procedure. *b*, summary of the blockade of the serotonin-induced increase in long-term facilitation by CRE injection. The height of each bar is the percentage change in the e.p.s.p. amplitude ± s.e.m. re-tested 24 h after treatment. (A two-tailed *t*-test comparison of means indicated that the decrease in e.p.s.p. in cultures injected with CRE oligonucleotide is significantly different ($P < 0.05$) from the increase in the e.p.s.p. in the cells injected with either the mutant or NF-$_\kappa$B oligonucleotides.) *c*, Summary of the pooled data for short-term facilitation 24 h after injection. In contrast to long-term facilitation, the serotonin (5 μM) was applied after the e.p.s.p. was first depressed. Five stimuli were given with an interstimulus interval of 30 s, and this resulted in 70–80% depression in e.p.s.p. amplitude. This serotonin now produced an increase in e.p.s.p. amplitude by the seventh stimulus. The increase in short-term facilitation was measured by calculating the percentage increase in the seventh e.p.s.p. amplitude as compared with the fifth e.p.s.p. amplitude. Because the facilitation here was of a depressed e.p.s.p., the percentage facilitation is larger than the long term, where only the non-depressed e.p.s.p. was examined.

METHODS. Standard electrophysiological techniques were used for recording from the cultured neurons[2]. During each recording session, the motor cell was impaled with a glass electrode (20–30 MΩ) filled with 3 M potassium acetate. The cells were recorded in a medium of equal volumes of artificial sea water and L15 culture medium (Flow Laboratory). *Aplysia* haemolymph was then added to a final concentration of 5% and the medium was filter sterilized.

the CRE oligonucleotide into the nucleus of the R2 neuron, which is sufficiently large for us to examine the heat-shock response in the same cell. As the volume of R2 is about 100 times greater than that of the sensory neurons, we injected a correspondingly greater amount of oligonucleotide. Induction of the family of heat-shock proteins was not affected, suggesting that the CRE oligonucleotide injection does not cause a general inhibition of transcription. (Similarly, injection of digested PKSM13$^+$ plasmid DNA (average size 128 nucleotides), or of the vehicle, failed to block the increase in synaptic strength in the sensory neurons.)

Our results, based on mobility shift and western blot assays, indicate that the nervous system of *Aplysia* contains a CREBP-like protein of relative molecular mass 43K. Moreover, the selective blockade of long- but not short-term facilitation by the binding of the CREBP, in a sequence-specific manner, implicates CREBP as one component of the molecular machinery for setting up long-term facilitation. Although the sensory neuron–motor neuron system still precludes a direct examination of the mechanism by which binding of CREBP blocks long-term facilitation, titration of CREBP by excess CRE in 3T3 cells blocks induction of cAMP-inducible genes[21]. We therefore think it likely that the CRE oligonucleotide works by inhibiting the action of CREBP, either on cAMP-inducible genes or on constitutively expressed genes that encode proteins that turn over rapidly[22]. It is also possible that the oligonucleotide works by titrating a general transcription factor that binds to CREBP, however we think this is less likely as injection of the HSE oligonucleotide does not block long-term facilitation.

These experiments also provide additional support (see refs 4, 5) for the importance of the cAMP pathway in initiating long-term facilitation. The data do not exclude, however, the possibility that other second messenger systems also participate in the long-term process. Indeed, we and others have found that CREBP can be phosphorylated by the C kinase, by calcium-calmodulin-dependent protein kinase II, and by casein kinase II (unpublished results) as well as by the A kinase. Moreover, CREBP is probably not the only protein factor important for the induction of the long-term process.

Nonetheless, as binding of CREBP seems to be an early step in long-term facilitation, and is a target for both cAMP-dependent and other kinases, purification of CREBP from *Aplysia* and characterization of the upstream region to which CREBP might bind in genes induced by serotonin should allow further study of the causal steps involved in the induction of the long-term process. Moreover, the approach used here to block gene function by intranuclear oligonucleotide injection might be generally useful in studying gene action in nerve cells. □

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